

The Role of Biomethylation in Toxicity and Carcinogenicity of Arsenic: A Research Update

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Recent research of the metabolism and biological effects of arsenic has profoundly changed our understanding of the role of metabolism in modulation of toxicity and carcinogenicity of this metalloid. Historically, the enzymatic conversion of inorganic arsenic to mono- and dimethylated species has been considered a major mechanism for detoxification of inorganic arsenic. However, compelling experimental evidence obtained from several laboratories suggests that biomethylation, particularly the production of methylated metabolites that contain trivalent arsenic, is a process that activates arsenic as a toxin and a carcinogen. This article summarizes this evidence and provides new data on *a*) the toxicity of methylated trivalent arsenicals in mammalian cells, *b*) the effects of methylated trivalent arsenicals on gene transcription, and *c*) the mechanisms involved in arsenic methylation in animal and human tissues. **Key words:** AP-1, arsenic, cancer, inhibition, methylated arsenic, methylation, methyltransferase, toxicity, transcription control. *Environ Health Perspect* 110(suppl 5):767–771 (2002).

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The metabolism of inorganic arsenic (iAs) in humans involves two types of chemical reactions, the reduction of pentavalent arsenicals to trivalency and the oxidative methylation of trivalent arsenicals to yield methylated pentavalent metabolites (1) (Figure 1). Glutathione (GSH) has been shown to reduce pentavalent arsenicals (arsenate iAs^V), methylarsonic acid (MAs^V), and dimethylarsinic acid (DMAs^V) in aqueous solutions (2,3). As^V reductases may enzymatically reduce these arsenicals in mammalian tissues (4,5). Methylation of trivalent arsenicals [arsenite (iAs^{III}) and methylarsonous acid (MAs^{III})] is catalyzed by As^{III}-methyltransferases that use S-adenosylmethionine (AdoMet) as the methyl group donor (6,7). Because MAs^V and DMAs^V are not toxic in acute lethality assays, methylation of iAs has long been considered a detoxification mechanism. However, methylated arsenicals that are chemically consistent with trivalent methylated metabolites, MAs^{III} and dimethylarsinous acid (DMAs^{III}), have been shown to be more potent enzyme inhibitors and cytotoxins than either iAs^V or iAs^{III} (8). Diiodomethylarsine (MAs^{III}I₂) and methylarsine oxide (MAs^{III}O) are potent inhibitors of glutathione disulfide (GSSG) reductase (9), pyruvate dehydrogenase (10), and especially thioredoxin reductase (11). MAs^{III}O and MAs^{III}I₂ are also far more toxic than iAs^{III} for various types of mammalian cells (12–14). DMAs^{III} derivatives [iododimethylarsine (DMAs^{III}I) and dimethylarsinous glutathione (DMAs^{III}GS)] are at least as cytotoxic as iAs^{III} for most cell types examined.

Notably, exposures to low concentrations of either MAs^{III}O or DMAs^{III}I induce cell proliferation and production of growth-promoting cytokines in normal human keratinocytes (NHEK) (15). Unlike iAs^{III} and iAs^V, MAs^{III}O and DMAs^{III}I react directly with DNA, nicking naked DNA *in vitro* and damaging nuclear DNA in intact human leukocytes (16). Evidence for the formation of methylated trivalent arsenicals in the course of the metabolism of iAs in humans has been obtained using optimized analytical techniques (17,18). MAs^{III} and DMAs^{III} have been detected in urine of individuals chronically exposed to iAs in drinking water (5,18–20) and in cultured human hepatic cells exposed to various concentrations of iAs^{III} (18). Studies are currently under way in several laboratories to elucidate the role of methylated trivalent metabolites in the systemic toxicity and carcinogenicity of iAs. This report summarizes some recent work linking the metabolism of arsenic to its biological effects.

Toxicity of Methylated Trivalent Arsenicals in Mammalian Cells

Cytotoxic effects of trivalent and pentavalent arsenicals have previously been examined in several cell types, including primary human hepatocytes, primary human bronchial epithelial cells (HBEC), NHEK, SV-40-immortalized human bladder epithelial (UROtsa) cells, HeLa cells (12,13), and Chang liver cells (14). Pentavalent arsenicals were significantly less cytotoxic than their trivalent counterparts (12–14). Among trivalent arsenicals, MAs^{III}O

and MAs^{III}I₂ were the most cytotoxic species, followed by DMAs^{III}I, DMAs^{III}GS, and iAs^{III}. We have recently examined cytotoxicity of arsenicals in several other mammalian cell types, including human hepatocellular carcinoma (HepG2) cells, human bladder transient carcinoma (T24) cells, human acute promyelocytic leukemia (NB4) cells, human monoblastoid (U937) cells, human osteosarcoma (HOS) cells, human neuroblastoma (SK-N-SH) cells, mouse 3T3 adipocytes, primary guinea pig hepatocytes, and Chinese hamster lung (V79-4) cells (Table 1). Regardless of the cell type, trivalent monomethylated arsenicals, MAs^{III}O and MAs^{III}I₂, were the most potent cytotoxins, with LC₅₀ values ranging from 0.4 to 5.5 μM. DMAs^{III} derivatives were as cytotoxic as MAs^{III} species and more cytotoxic than iAs^{III} in most cell types.

The thiazolyl blue (MTT) assay that monitors the activity of mitochondrial dehydrogenases in viable cells has been used to examine cytotoxicity of arsenicals in all these cell types. The neutral red assay that measures the uptake of the dye by viable cells has also been used in some experiments. Because the cell viability values determined by the neutral red assay were lower than those obtained by the MTT

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assay (12,15), it is possible that the latter assay underestimates cytotoxic effects of arsenicals in cultured cells. Figure 2 shows an example of the concentration-dependent effects of trivalent arsenicals on cell viability in human leukemia NB4 and U937 cell lines. Increased cell viability values found after 24-hr exposures to low concentrations of arsenicals were associated with increased cell proliferation rates. The induction of cell proliferation by low concentrations of trivalent arsenicals has previously been reported in several cell types [e.g., NHEK (15)]. Notably, among cell types examined, NB4 cells were most sensitive to cytotoxic effects of trivalent arsenicals.

As shown in Table 1, there was no apparent correlation between the capacity of cells to methylate iAs and their sensitivity to the cytotoxic effects of trivalent arsenicals, indicating that the capacity to methylate has little to do with the resistance of cells to acute toxicity of As^{III}. In some cases, iAs^{III} was more toxic in cells with a high methylation capacity (e.g., rat hepatocytes) than in cells that do not methylate this arsenical (e.g., guinea pig hepatocytes). Consequently, mechanisms other than methylation (e.g., transport of arsenicals across the cell membrane or protein binding) may play a critical role in the detoxification of trivalent arsenicals under acute exposure conditions. These results suggest that production and accumulation of MAs^{III} and/or DMAs^{III}, the most cytotoxic species among biologically relevant arsenicals, may be directly linked to adverse effects associated with *in vivo* exposures to iAs. We have previously shown that HepG2 cells exposed to iAs^{III} produced both MAs^{III} and DMAs^{III}. In addition, both MAs^{III} and DMAs^{III} synthesized in HepG2 cells were released into culture medium (18). Hence, MAs^{III} and DMAs^{III} may be translocated from methylating cells to tissues and cells that cannot methylate iAs. Notably, production of

MAs^{III} and DMAs^{III} by HepG2 cells increased with increasing concentrations of iAs^{III} in the culture. Similarly, epidemiologic studies have shown that urinary levels of MAs^{III} and DMAs^{III} in individuals exposed to iAs in drinking water are positively correlated with exposure levels (5,18). These results suggest that individuals exposed to higher levels of iAs may be at greater risk associated with the production of these toxic methylated metabolites.

Effects of Methylated Trivalent Arsenicals on Gene Transcription

Various hypotheses have been proposed to explain the carcinogenicity of iAs (28). Nevertheless, molecular mechanisms by which this arsenical induces cancer are still poorly understood. Results of previous studies indicated that iAs does not act through classic genotoxic and mutagenic mechanisms, but

rather may be a tumor promoter that modifies signal transduction pathways involved in cell growth and proliferation (29). iAs^{III} has been shown to modulate expression and/or DNA-binding activities of several key transcription factors, including nuclear factor kappa B (30), tumor suppressor 53 (p53) (31), and activating protein-1 (AP-1) (32–34). Mechanisms of AP-1 activation by iAs^{III} include stimulation of the mitogen-activated protein kinase (MAPK) cascade with a consequent increase in the expression and/or phosphorylation of the two major AP-1 constituents, c-Jun and c-Fos (29). The modulation of AP-1-dependent gene transcription by iAs^{III} may contribute to the induction of cell proliferation in cultured cells exposed to this arsenical. However, there are no data on the effects of methylated trivalent arsenicals on AP-1 composition and DNA-binding activity.

Recently, we have examined c-Jun and c-Fos expression and AP-1 DNA-binding

Table 1. Toxicity of trivalent arsenicals in mammalian cells.

Cell type	Estimated LC ₅₀ values ^a (μM)					Methylation rate ^b (pmol As/10 ⁶ cells/hr)	References
	iAs ^{III}	MAs ^{III} O	MAs ^{III} I ₂	DMAs ^{III} GS	DMAs ^{III} I		
Primary rat hepatocytes	10–>20	2.8	1.8	14.5	2.7	19	(12,13)
Primary human hepatocytes	20	5.5		>20		3.3	(13)
NHEK	10–>20	2.6		8.5		0.12	(12,13)
HBEC	3.2	2.7		6.8		0.05	(13)
UROtsa	17.8	2.0	0.8	14.2	>20	ND ^c	(12,13)
HeLa	>20	2.8		>20		0.25	(12)
HepG2	>50	4.0			8.0	0.5	(21)
T24	>10	2.5			>10	ND	(21)
NB4	1.0	0.4			0.4	ND	(22)
U937	5.0	1.0			1.0	ND	(22)
HOS	25	1.6			7.0	0.25	(23)
SK-N-SH	40	2.7			2.0	ND	(24)
3T3adipocytes	100	5.0			6.0	0.3	(25)
Primary guinea pig hepatocytes	50	3.2			5.0	ND	(26)
V79	4.5	0.5			0.4	ND	(27)

Abbreviations: ND, not determined. ^aLC₅₀ is defined as the concentration of an arsenical that resulted in a 50% decrease in cell viability over a 24-hr incubation period. The MTT assay was used to examine cell viability in all cell types. ^bMethylation rates were determined in cultures exposed to 0.05 or 0.1 μM iAs^{III} for 24 or 48 hr. ^cMethylation activity not detected.

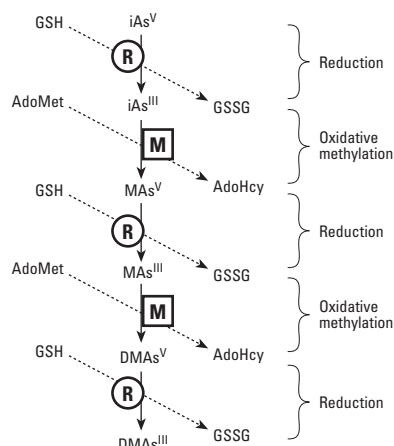


Figure 1. Scheme of the metabolic conversions of iAs in humans. AdoHcy, S-adenosylhomocysteine; R, As^V reductase; M, As^{III} methyltransferase.

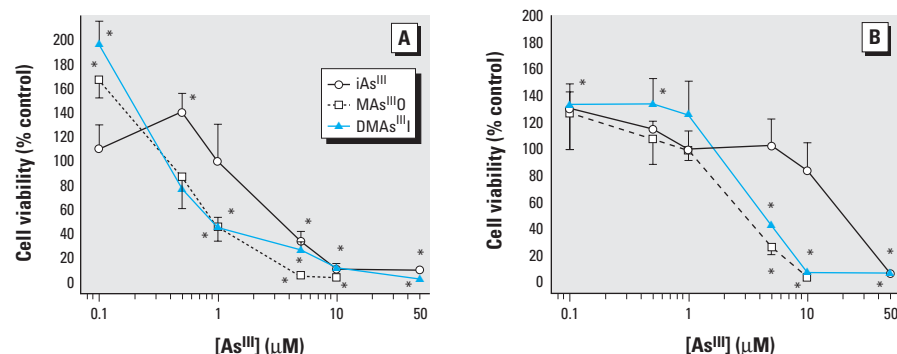


Figure 2. Effects of trivalent arsenicals on cell viability in (A) NB4 and (B) U937 cultures. Cell viability was determined by the MTT assay after 24-hr exposures to iAs^{III} (circles), MAs^{III}O (squares), or DMAs^{III}I (triangles). Each symbol and error bar represents mean and SD for *n* = 4. Asterisk (*) indicates cell viability in treated cultures is significantly different (*p* < 0.05) from that in untreated cultures as determined by analysis of variance with the Dunnett multiple comparison posttest.

activity in several human cell lines, including UROtsa, T24, HepG2, and primary human hepatocytes exposed to trivalent or pentavalent inorganic or methylated arsenicals. Short-time exposures to trivalent, but not to pentavalent, arsenicals increased AP-1 DNA-binding activity in all these cell types. Most profound effects were found in UROtsa and T24 cells. In these cell lines, exposures to $\text{MAs}^{\text{III}}\text{O}$ or $\text{DMAs}^{\text{III}}\text{I}$ significantly increased the levels of nuclear phospho-c-Jun (p-c-Jun) but had no effects on either c-Jun or c-Fos levels (35). Importantly, $\text{MAs}^{\text{III}}\text{O}$ and $\text{DMAs}^{\text{III}}\text{I}$ were considerably more potent inducers of c-Jun phosphorylation and AP-1 activation than was iAs^{III} . Neither iAs^{V} nor

methylated pentavalent arsenicals, MAs^{V} or DMAs^{V} , modified c-Jun phosphorylation. Figure 3 shows nuclear levels of p-c-Jun in UROtsa cells exposed for 1 hr to iAs^{III} , $\text{MAs}^{\text{III}}\text{O}$, or $\text{DMAs}^{\text{III}}\text{I}$ (0.5, 1, or 5 μM). $\text{MAs}^{\text{III}}\text{O}$ was the most potent inducer of p-c-Jun, followed by $\text{DMAs}^{\text{III}}\text{I}$. In contrast, exposures to iAs^{III} suppressed p-c-Jun levels in this cell line. The AP-1 DNA-binding activity was induced in UROtsa cells exposed to as little as 0.1 μM $\text{MAs}^{\text{III}}\text{O}$ (35), a concentration that is well below the LC_{50} value for these cells (Table 1).

The potencies of $\text{MAs}^{\text{III}}\text{O}$ and $\text{DMAs}^{\text{III}}\text{I}$ to stimulate the AP-1-dependent gene transcription have further been demonstrated using UROtsa and T24 cells transiently transfected with an AP-1-dependent promoter-reporter construct (35). Somewhat different AP-1 activation patterns were observed in primary human hepatocytes (Figure 4). Among trivalent arsenicals examined, $\text{MAs}^{\text{III}}\text{O}$ was the most potent inducer of c-Jun phosphorylation in these cells. However, only a weak induction of p-c-Jun was observed in human hepatocytes exposed to iAs^{III} or $\text{DMAs}^{\text{III}}\text{I}$. A significant induction of the AP-1 DNA-binding activity was detected by the electrophoretic mobility shift assay (EMSA) only in cells exposed to

5 μM $\text{MAs}^{\text{III}}\text{O}$ (Figure 5). Under these exposure conditions, p-c-Jun, but not c-Fos, was the major constituent of the AP-1 DNA-binding complex. Based on these results, the AP-1 DNA-binding activity appears to be less sensitive to induction by trivalent arsenicals in primary human hepatocytes than in either UROtsa or T24 cell lines that are derived from human urinary bladder. Accordingly, trivalent arsenicals, particularly MAs^{III} , are likely to induce the AP-1-dependent gene transcription in human bladder to a greater extent than in the liver. Notably, both hepatic and urinary bladder cancers have been associated with chronic exposures to iAs in drinking water. However, the incidence of bladder cancer exceeds that of hepatic cancer (36–38). Thus, trivalent methylated arsenicals that are chemically consistent with trivalent methylated metabolites of iAs are more potent than iAs, inducing the DNA-binding activity of AP-1, a key transcription factor that is involved in regulation of cell proliferation and death (29).

Mechanism of iAs Methylation

The enzymatic reactions involved in the reduction and methylation of arsenicals have

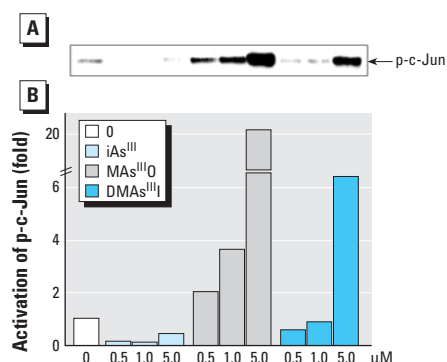


Figure 3. Immunoblot analysis of p-c-Jun in nuclear protein extracts from UROtsa cells exposed to iAs^{III} , $\text{MAs}^{\text{III}}\text{O}$, or $\text{DMAs}^{\text{III}}\text{I}$ for 1 hr and from control (untreated) cells. Nuclear proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electroblotted on polyvinylidene difluoride membranes. Membranes were blocked with 5% nonfat milk, washed, and probed with a mouse monoclonal antibody specific for p-c-Jun (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Blots were visualized by an enhanced chemiluminescence technology and quantified using a digital imaging system. Immunoblot images (A) and results of the quantitative analysis of these images (B) are shown.

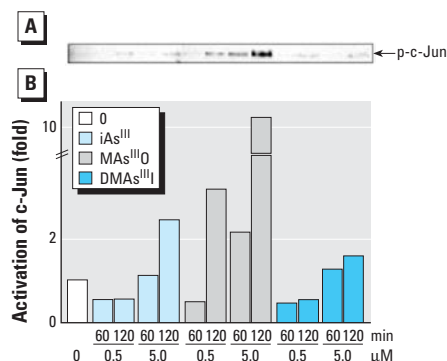


Figure 4. Immunoblot analysis of p-c-Jun in nuclear protein extracts from primary human hepatocytes exposed to iAs^{III} , $\text{MAs}^{\text{III}}\text{O}$, or $\text{DMAs}^{\text{III}}\text{I}$ for 1 or 2 hr and from control (untreated) cells. Immunoblot images (A) and results of the quantitative analysis of these images (B) are shown. For description of method, see Figure 3.

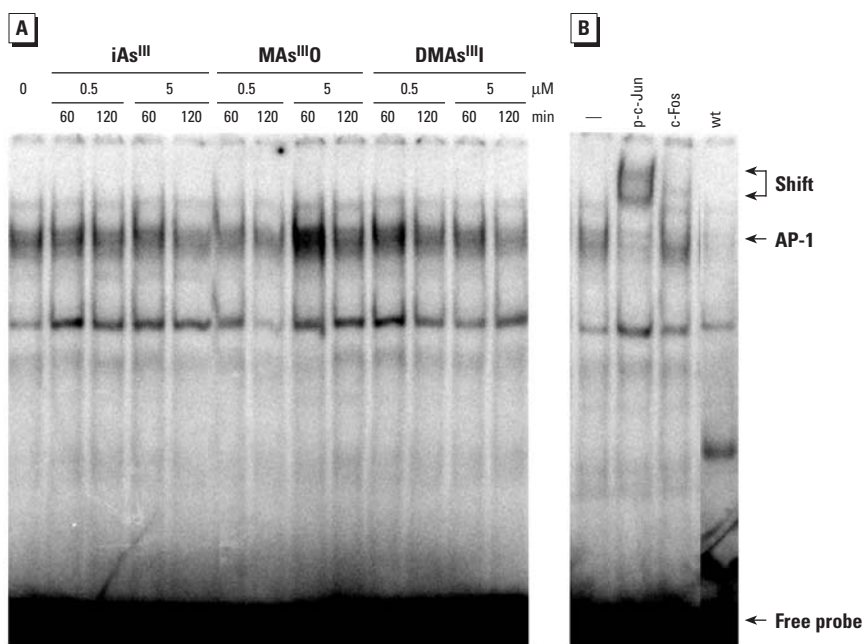


Figure 5. AP-1 DNA-binding activity in primary human hepatocytes treated with trivalent arsenicals and in control (untreated) cells. (A) EMSA of nuclear protein extracts from cells treated for 1 or 2 hr with iAs^{III} , $\text{MAs}^{\text{III}}\text{O}$, or $\text{DMAs}^{\text{III}}\text{I}$ and from control cells. The DNA–protein binding assay was performed in a reaction buffer (28) containing a radiolabeled AP-1-binding probe (5′-TGAGTCAG-3′; Promega, Madison, WI, USA), nuclear proteins, and poly(dI-dC) · poly(dI-dC) (Boehringer Ingelheim, Ridgefield, CT, USA). The DNA-binding complexes were separated by PAGE, and the distribution of radioactivity was analyzed in dried gels by phosphorimaging. (B) EMSA-supershift analysis of a nuclear protein extract from cells treated with 5 μM $\text{MAs}^{\text{III}}\text{I}$ for 1 hr. The reaction and analysis were performed as described for A. To identify the AP-1 dimer constituents, antibodies specific for p-c-Jun (lane 2) or for c-Fos (lane 3) (both from Santa Cruz Biotechnology) were added into the DNA–protein binding mixture. The specificity of the assay was established using a 50-fold excess of a wild-type (wt, lane 4) AP-1 probe.

been studied in several laboratories using fractionated tissues, intact cells, and purified enzymes (8). Distinct As^{V} reductases have been shown to catalyze reduction of iAs^{V} to iAs^{III} and of MAs^{V} to MAs^{III} (4,39). Both these enzymes require thiols (e.g., GSH) for reducing activity. The MAs^{V} reductase ($K_m = 2.6 \text{ mM}$) has recently been identified as GSH-S-transferase omega (40). Methyltransferases that catalyze methylation of iAs^{III} and MAs^{III} have also been identified. A rabbit liver enzyme that converts iAs^{III} to MAs and MAs^{III} to DMAs has been purified and partially characterized (6). This cytosolic protein has a molecular weight of about 60 kDa and requires both AdoMet and a thiol for activity. Consistent with the metabolic scheme in Figure 1, the purified enzyme has a greater affinity for MAs^{III} than for MAs^{V} .

A novel As^{III} methyltransferase ($M_r = 41 \text{ kDa}$) has recently been purified by Lin and co-workers (7) from rat liver. This enzyme methylates iAs^{III} in a two-step reaction, in which MAs is an intermediate and DMAs is the final product. The two-step kinetics of this reaction is consistent with kinetic patterns of iAs^{III} methylation reported in *in vitro* studies using tissue extracts (41,42). AdoMet is the essential methyl group donor for both methylation steps (Table 2). MAs^{III} is also a substrate for this enzyme in a methylation

reaction yielding DMAs . A kinetic analysis of this reaction showed a low K_m of 250 nM MAs^{III} . Thus, this enzyme can effectively methylate at very low concentrations of MAs^{III} in tissues. However, high concentrations of MAs^{III} ($\geq 5 \mu\text{M}$) inhibit DMAs synthesis. The rat As^{III} methyltransferase requires a dithiol for its activity. Dithiothreitol (DTT) has been used as an enzyme co-factor in *in vitro* assays with purified rat As^{III} methyltransferase. Protein and cDNA sequences for the rat As^{III} methyltransferase have been obtained. Sequence analyses have revealed a high degree of homology with a putative human methyltransferase CYT19, indicating that CYT19 is the human As^{III} methyltransferase. Using reverse-transcription polymerase chain reaction, mRNA for As^{III} methyltransferase has been detected in rat tissues (liver, heart, lung, kidney, adrenal, bladder, and brain) and also in human hepatoma (HepG2) cells that are known to methylate iAs^{III} (18). In contrast, mRNA for this enzyme has not been found in UROtsa cells, human urinary bladder cells that do not produce methylated metabolites when exposed to iAs^{III} in culture (12,13).

Based on results of the *in vitro* studies, the presence of a dithiol is an essential requirement for the rat As^{III} methyltransferase activity. Thioredoxin (TRx), a small (12 kDa) protein with a pair of redox-active cysteine residues

(44), is a likely candidate for the role of a cofactor for this enzyme in mammalian cells. In fact, TRx and DTT are equally effective in supporting the *in vitro* As^{III} methyltransferase activity (43). The main function of TRx in cells is the reduction of disulfide bonds in molecules of various proteins, including enzymes, cellular receptors, and transcription factors. During this reaction, the redox-active sulfhydryl groups in TRx molecule are oxidized to form a disulfide (45). The oxidized TRx is then reactivated in an NADPH-dependent reaction catalyzed by TRx-reductase (TR) (44).

The mechanism of interactions between TRx and As^{III} methyltransferase has not been examined. It is likely that TRx is involved in the reduction of the pentavalent intermediate, MAs^{V} . TRx may directly reduce MAs^{V} to MAs^{III} before the second methylation step. It may also be a donor of electrons for reduction (reactivation) of redox-active cysteinyl residues of the As^{III} methyltransferase that are responsible for MAs^{V} reduction. Alternatively, TRx may reduce other cysteinyl residues that are required for the catalytically active conformation of the enzyme. Notably, interactions between TRx and As^{III} methyltransferase provide a basis for a hypothetical mechanism that may play an important role in the regulation of this enzyme (Figure 6). It has been shown that MAs^{III} derivatives [MAs^{III} or $\text{MAs}^{\text{III}}\text{I}_2$] are potent inhibitors of TR (11), the enzyme responsible for TRx reactivation. In cell cultures exposed to iAs^{III} , inhibition of TR activity correlates with accumulation of MAs in cells (46). These data suggest that MAs^{III} , the intermediate formed in the course iAs methylation, is responsible for inhibition of TR activity. The inhibition of TR by MAs^{III} may result in a decreased availability of the active (reduced) form of TRx in cells, preventing further reduction of MAs^{V} to MAs^{III} . This hypothetical regulatory mechanism would retard the formation of MAs^{III} when the concentrations of this toxic intermediate in cells reached low micromolar values.

Conclusions

The results of previous studies and new experimental data presented here suggest that exposures to methylated trivalent arsenicals are associated with a variety of adverse effects that have a profound impact on cell viability or proliferation. The known effects include *a*) inhibition of several key enzymes, *b*) damage to DNA structure, and *c*) activation of AP-1-dependent gene transcription. Notably, trivalent methylated arsenicals, MAs^{III} and/or DMAs^{III} derivatives, are more potent than iAs^{III} in producing these effects. These findings are consistent with the concept of biomethylation

Table 2. Properties of As^{III} methyltransferase from rat liver.^a

Molecular mass	42,000 Da (determined by PAGE); 41,056 Da (calculated)
Primary structure characteristics	369 amino acid residues; cysteine-rich protein (12 cysteine residues); common methyltransferase motifs, including the AdoMet binding motifs
Kinetic characteristics	$K_m = 250 \text{ nM}$, $V_{\text{max}} = 68 \text{ pmol/mg protein/min}$ [with MAs^{III} as a substrate]; pH optimum = 9.5 (active from pH 7 to 11)
Essential co-factors	Dithiol (e.g., DTT, TRx); AdoMet (the methyl group donor)
Known inhibitor	MAs^{III} ($\geq 5 \mu\text{M}$) inhibits DMAs formation in the second methylation reaction

^aData from Lin et al. (7) and Lin and Thomas (43).

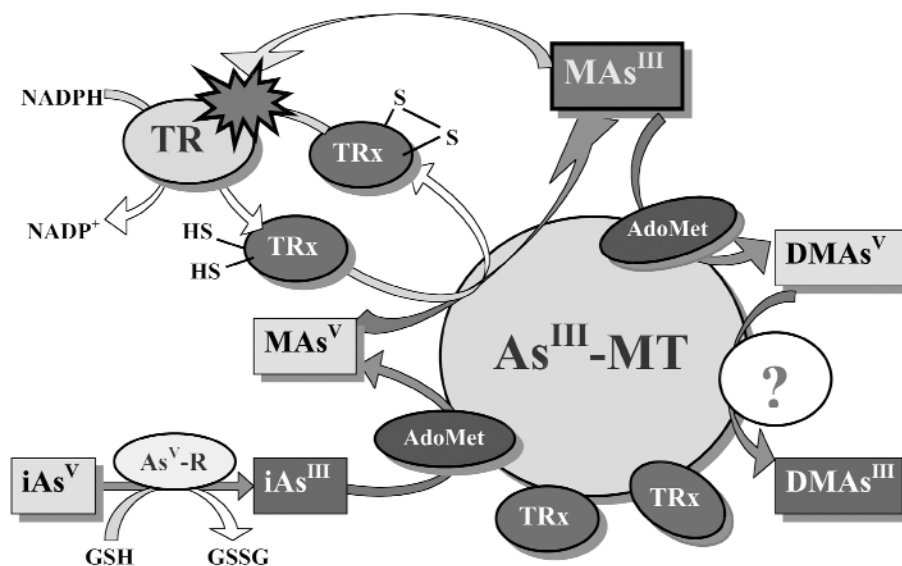


Figure 6. Hypothetical mechanism of the methylation of iAs by As^{III} methyltransferase: the role of TRx and TR. As^{V} -R, As^{V} reductase; As^{III} -MT, As^{III} methyltransferase.

tion being a process that potentiates toxicity and carcinogenicity of iAs.

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